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Effects of Selective Prostaglandin EP4 Receptor Antagonist on Osteoclast Formation and Bone Resorption In Vitro

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Prostaglandin estradiol (PGE_2) stimulates bone resorption by a cyclic AMP (cAMP)-dependent mechanism that involves prostaglandin E receptors of the EP2 and EP4 subtypes. We tested a potent selective EP4 antagonist (EP4RA), which blocks PGE_2 binding to EP4 receptors. We examined the effects of EP4RA on osteoclastogenesis in murine marrow cultures, on cAMP production in primary osteoblastic (POb) cell cultures, and on bone resorption in organ cultures. EP4RA (1 μmol/L) decreased the number of tartrate-resistant acid phosphatase-positive multinucleated cells ($TRAP^+$ MNC) by 46%–48% in cultures treated with 0.1–1.0 μmol/L PGE_2 and by 96% in cultures treated with 0.01 μmol/L PGE_2. EP4RA also decreased $TRAP^+$ MNC formation by 60% in 1,25-dihydroxyvitamin D (1,25D)-treated cultures and by 62% in parathyroid hormone (PTH)-treated cultures. A chemically related analog of EP4RA that lacks antagonist activity did not inhibit $TRAP^+$ MNC formation. EP4RA decreased cAMP production in PGE_2-treated POb by 44% but did not block cAMP response to PTH. EP4RA inhibited the increase in receptor activator of NF-κB ligand (RANKL) mRNA levels produced by PGE_2. In fetal rat long bone cultures, EP4RA decreased ^{45}Ca release from control, unstimulated cultures by 12%–25% and from PGE_2-stimulated cultures by 22%–37%. Because EP4RA partially inhibited osteoclastogenesis not only in response to PGE_2 but also in response to 1,25D and PTH, these results suggest that activation of the EP4 receptor may play a general role in osteoclastic bone resorption. EP4RA showed partial inhibition of PGE_2-stimulated osteoclastogenesis at 1 μmol/L, but almost complete inhibition at 0.01 μmol/L PGE_2. This could be due to the limited efficacy of the antagonist at high concentrations of PGE_2, or an alternative pathway, such as activation of the EP2 receptor. (Bone 30:159–163; 2002)
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Key Words: Prostaglandin estradiol (PGE_2); EP4 receptor; Osteoclastogenesis; Bone resorption; cAMP.

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Introduction

Prostaglandins (PGs) are complex, multifunctional regulators of bone metabolism.[13,14] Prostaglandin E_2 (PGE_2) is a potent bone resorber and is produced in bone mainly by osteoblasts. The multiple effects of PGs are mediated through specific cell surface receptors coupled to G proteins. Effects of PGE_2 are mediated by EP1, EP2, EP3, and EP4 receptors.[5] PGE_2 stimulates bone resorption by a cyclic AMP (cAMP)-dependent mechanism. Among the PGE receptors, EP2 and EP4 stimulate adenylylcyclase, hence the EP2 and EP4 receptors are most likely to mediate the stimulatory effects of PGE_2 on bone resorption. Bone cells and marrow stromal cells can express both EP2 and EP4 receptors.[6,10,16] PGE_2 also promotes osteoclast-like cell formation in mouse bone marrow cultures.[2,18] It was previously reported that the EP4 antagonist, AH23848B, inhibited PGE_2-induced osteoclast formation in bone marrow culture.[12] Moreover, spleen and marrow cells have a diminished capacity to form osteoclast-like cells in cocultures with osteoblasts lacking the EP4 receptor ($EP4^{-/-}$).[15] Organ cultures of $EP4^{-/-}$ calvariae have shown diminished resorption and metalloproteinase production in response to PGE_2.[9]

On the other hand, we recently reported decreased osteoclast formation when either osteoblastic or osteoclastic precursor cells were derived from animals lacking the EP2 receptor.[7] Using specific agonists for EP2 and EP4 receptors, Suzawa et al.[17] suggested that PGE_2 stimulates bone resorption by a mechanism involving cAMP and receptor activator of NF-κB ligand (RANKL), which is mediated by both EP4 and EP2. PGE_2 may also play a role in the stimulation of tartrate-resistant acid phosphatase-positive mononuclear cell ($TRAP^+$ MNC) formation by other agents such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 (1,25D). We recently found that marrow cultures from animals lacking inducible cyclooxygenase (COX-2) showed decreased $TRAP^+$ MNC formation in response to PTH and 1,25D, which can be reversed by addition of PGE_2.[11]

In the present study, we used a potent EP4-receptor-selective antagonist, L-161,982 (EP4RA), and its inactive analog, L-161,983 (analog), to further test the role of the EP4 receptor in osteoclast formation and activation. EP4RA inhibited formation of $TRAP^+$ MNC stimulated by PGE_2 in bone marrow cultures and cAMP production and RANKL expression in primary mouse osteoblastic cell (POb) cultures. EP4RA also inhibited stimulation of $TRAP^+$ MNC formation by PTH and 1,25D. In addition, EP4RA partially inhibited bone resorption stimulated by PGE_2 in rat long bone cultures.

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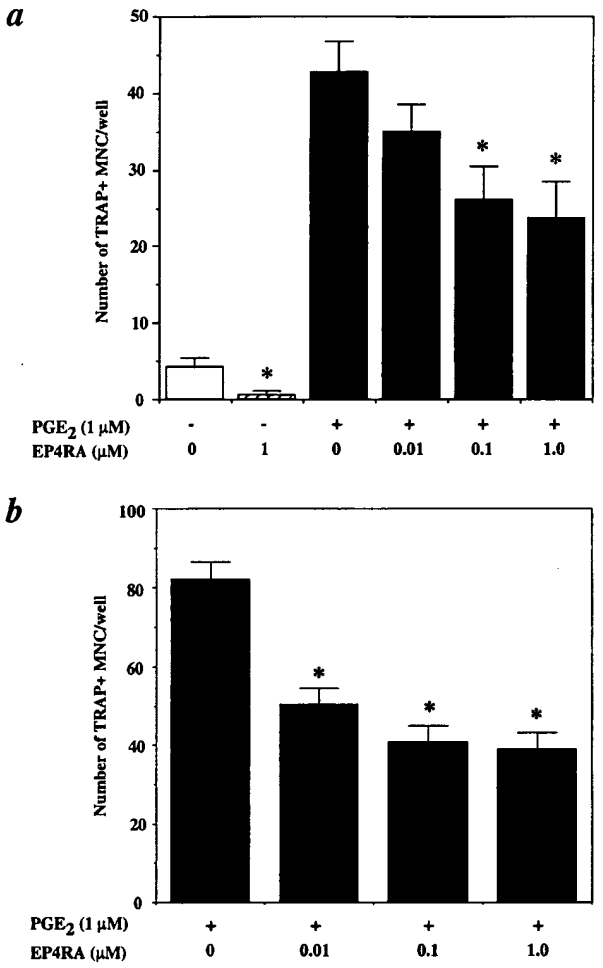

Figure 1. Dose-dependent effects of EP4RA on TRAP$^+$ MNC formation in bone marrow culture stimulated by PGE_2. Bone marrow cells were treated with PGE_2 (1 μmol/L), and EP4RA (0.01–1 μmol/L). Results from two representative experiments are shown (a, b). Columns are means and vertical lines, SEM (n = 6). *Significant effect of EP4RA ($p < 0.05$).

Materials and Methods

Animals and Reagents

For bone marrow cultures we used 4–12-week old C57B1/6 mice (Charles River Farms, Wilmington, MA). For long bone resorption assays we used timed-pregnant Sprague–Dawley rats (Charles River Farms). L-161982 (5-butyl-2,4-dihydro-4-[[2'-[*N*-(5-methyl-2-thiophenecarbonyl)sulfamoyl]biphenyl-4-yl]methyl]-2-[(2-trifluoromethyl)phenyl]-1,2,4-triazol-3-one) and a chemically related inactive analog, L-161983, were kindly provided by Dr. R. N. Young (Merck Frosst Canada, Inc.). L-161983 was originally synthesized as an angiotensin-receptor antagonist.[3] The two compounds differ only by the position of a methyl group on the thiophene ring. The IC_{50} of EP4RA for the EP4 receptor is 30 nmol/L, whereas the IC_{50} for the EP2 receptor is 58,000 nmol/L, indicating over 1000-fold selectivity. PGE_2, PTH, and other chemicals were purchased from Sigma (St. Louis, MO). 1,25-dihydroxyvitamin D_3 (1,25D) was purchased from Biomol (Plymouth Meeting, PA).

Bone Marrow Cultures

Marrow was flushed from femora and tibiae of mice and 2–5 × 10^6 cells/well were plated in 24 well culture dishes and cultured in 0.5 mL of α-minimal essential medium (α-MEM) without phenol red (Gibco-BRL; McLean, VA) containing 10% heat-inactivated fetal calf serum (FCS, Gibco-BRL) for 7 days. Eighty percent of the medium was replaced on days 3 and 5. Cultures were treated with or without EP4RA, analog, PGE_2, PTH, or 1,25D. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. On day 7, cells were fixed with 2.5% glutaraldehyde and TRAP stained using a staining kit. TRAP$^+$ MNC were counted using an inverted microscope (×200). The cells that stained TRAP$^+$ in the cytoplasm and had at least three nuclei were counted as TRAP$^+$ MNC.

Primary Osteoblastic Cells (POb) and Measurement of Intracellular cAMP Level

Whole calvariae were excised from 6-week-old C57B1/6 mice or neonatal C57B1/6 × 129 Sv mice (Charles River Farms), rinsed in α-MEM, and digested sequentially with 0.5 mg/mL crude Collagenase P (Boehringer Mannheim, Indianapolis, IN) and 20% trypsin-ethylene diamine tetraacetic acid (EDTA) (Gibco-BRL). Five digests were performed, all for 10 min, except the last one, which was for 30 min. Released cells were removed, the reaction was stopped with 10% FCS, and the solution was filtered through a Nitex membrane, (Millipore Corp., Bedford, MA) to ensure a single cell suspension. Digests 2–5 were pooled and plated in six well dishes (Costar) at 5000 cells/cm^2 in the same medium used for bone marrow culture. After 6 or 7 days of culture (cells were preconfluent), medium was changed to α-MEM containing 0.1% bovine serum albumin and 0.5 mmol/L isobutylmethylxanthine (IBMX), with or without 1 μmol/L EP4RA (2 mL of medium/well). After 20 min, cells were treated with PGE_2 (1 μmol/L or 0.1 μmol/L), or PTH (10 nmol/L) for 15 min. Medium (1.8 mL) was removed and cells were scraped into 400 μL of ice-cold ethanol. The ethanolic cell suspension was collected in a microcentrifuge tube and centrifuged to remove debris. Supernatants were evaporated to dryness and cAMP was measured using an EIA kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's protocol.

RNA Extraction and Northern Analysis

Total RNA was extracted from cultured POb by the method of Chomczynski and Sacchi.[4] Briefly, cells were homogenized in TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) extracted with chloroform, and RNA precipitated with isopropanol and washed with 80% ethanol. After quantitation at 260 nm, 20 μg of total RNA was run on the 1% agarose solidus/2.2 mol/L formaldehyde gel and transferred to a nylon membrane (Genescreen, New England Nuclear, Boston, MA) by capillary blotting and fixed to the membrane by ultraviolet (UV) irradiation. After 2 h of prehybridization in a 50% formamide solution at 42°C, filters were hybridized overnight in a similar solution in rotating cylinders at 42°C with a random primer [^{32}P]dCTP-labeled (NEN Life Science Products, Inc., Boston, MA) cDNA probe for mouse RANKL (obtained from Dr. Dirk Anderson, Immunex Corp., Seattle, WA) and mouse osteoprotegerin (OPG; obtained from Dr. W. Boyle, Amgen, Inc., Thousand Oaks, CA). Murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified by polymerase chain reaction (PCR) using a mouse GAPDH control amplimer set from Clontech (Palo Alto, CA). Filters were washed once in a 1 × SSC, 1% sodium dodecylsulfate (SDS) solution at room temperature, once in

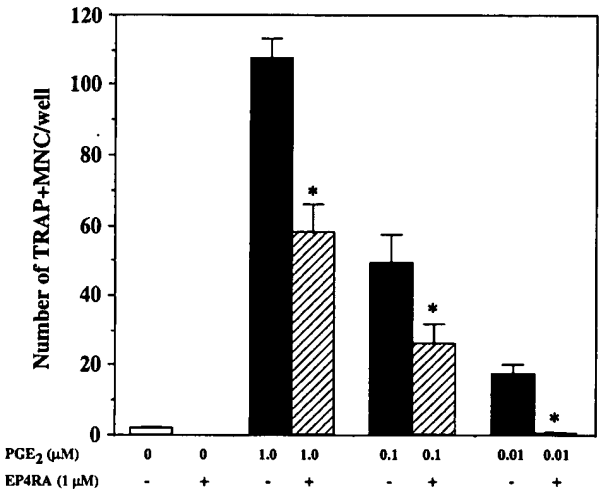

Figure 2. Effects of different concentrations of PGE_2 on $TRAP^+$ MNC and the response to EP4RA in bone marrow cultures. Bone marrow cells were treated with PGE_2 (0.01–1 μmol/L) with or without EP4RA (1 μmol/L). Columns are means and vertical lines, SEM (n = 6). *Significant effect of EP4RA ($p < 0.05$).

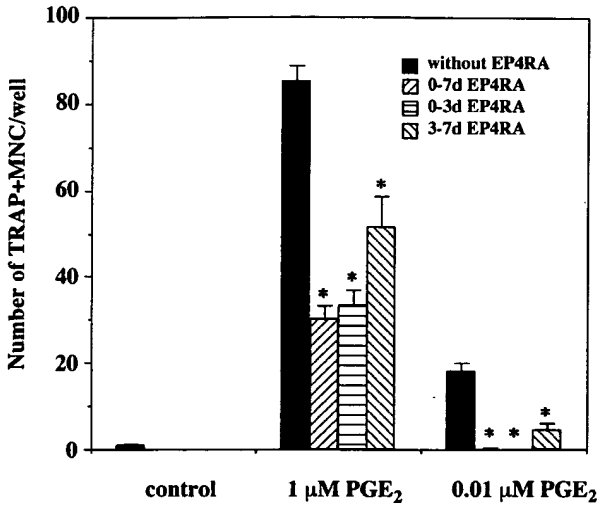

Figure 3. Effects of EP4RA (1 μmol/L) for different times in culture on $TRAP^+$ MNC formation in bone marrow culture. Bone marrow cells were treated with PGE_2 (1 or 0.01 μmol/L) with or without EP4RA (1 μmol/L) for 0–7, 0–3, or 3–7 days. Columns are means and vertical lines (SEM; n = 6). *Significant effect of EP4RA ($p < 0.05$).

0.1 × SSC, 0.1% SDS solution at 65°C. After washing, the filter was exposed to Kodak (Rochester, NY) XAR-5 film at −80°C. Filters were hybridized with one cDNA at a time and stripped with boiling 0.1% SDS between hybridization. Signals were quantitated densitometrically by NIH IMAGE v1.62 software. Optical densities for RANKL and OPG were normalized to the corresponding values for GAPDH.

Fetal Long Bone Resorption Assay

Eighteen day pregnant rats were injected with 200 μCi of ^{45}Ca (NEN). On day 19, rats were killed and the fetuses were removed. Radii and ulnae were dissected free of cartilage and soft tissue and cultured in α-MEM with 1 mg/mL bovine serum albumin (RIA grade, Sigma) for 5 days with a medium change after 2 days. ^{45}Ca in the bones and medium was counted and the percent of ^{45}Ca released was calculated.

Statistical Analysis

Means of groups were compared by analysis of variance (ANOVA), and significance of differences was determined by the Bonferroni *t*-test. Significance of differences for percent inhibition was determined by *t*-test.

Results

EP4RA caused a dose-related inhibition of PGE_2-stimulated $TRAP^+$ MNC formation in bone marrow culture. EP4RA at 0.01, 0.1, and 1 μmol/L inhibited $TRAP^+$ MNC formation stimulated by PGE_2 (1 μmol/L) by 18%, 39%, and 45%, respectively, in one experiment (**Figure 1a**) and by 39%, 50%, and 52%, respectively, in a second experiment (Figure 1b). PGE_2 (0.01–1 μmol/L) produced a dose-related increase in the number of $TRAP^+$ MNC in murine bone marrow culture (**Figure 2**). EP4RA (1 μmol/L) caused a 96%–99% inhibition of the response to a low dose (0.01 μmol/L) of PGE_2 (Figure 2 and **Figure 3**). EP4RA also inhibited the low but measurable number of $TRAP^+$ MNC formed in control cultures (Figure 1 and **Table 1**). The time-dependent effects of EP4RA on $TRAP^+$ MNC formation stimulated by PGE_2 were examined by treating with 1 μmol/L EP4RA for 0–7, 0–3, or 3–7 days (Figure 3). In cultures treated with 1 μmol/L PGE_2, 0–7 days of EP4RA inhibited $TRAP^+$ MNC formation by 65%, 0–3 days of EP4RA inhibited $TRAP^+$ MNC formation by 61%, and 3–7 days of EP4RA inhibited $TRAP^+$ MNC formation by 40%. In cultures treated with 0.01 μmol/L PGE_2, 0–7 days of EP4RA inhibited $TRAP^+$ MNC formation by 99%, 0–3 days of EP4RA inhibited $TRAP^+$ MNC formation by 100%, and 3–7 days of EP4RA inhibited $TRAP^+$ MNC formation by 76%. The inactive analog of EP4RA did not affect $TRAP^+$ MNC formation stimulated by 1 μmol/L PGE_2 in this system (**Figure 4**).

EP4RA also inhibited $TRAP^+$ MNC formation stimulated by 1,25D (10 nmol/L) by 60 ± 4%, and $TRAP^+$ MNC formation stimulated by PTH (10 nmol/L) by 62 ± 13% (Table 1). The analog did not inhibit $TRAP^+$ MNC formation in PTH or 1,25D-treated cultures (data not shown).

We examined the effects of EP4RA on cAMP production by primary osteoblastic cells (POb). EP4RA (1 μmol/L) inhibited cAMP production stimulated by PGE_2 (1 μmol/L) by 44%, but EP4RA did not inhibit cAMP production stimulated by PTH (10 nmol/L) (**Figure 5**). In separate experiments, we used POb from

Table 1. Inhibition by EP4RA of $TRAP^+$ MNC formation stimulated by 1,25-vitamin D_3 (10 nmol/L), or parathyroid hormone (10 nmol/L)

Treatment	Number of experiments	$TRAP^+$ MNC without EP4RA	$TRAP^+$ MNC with EP4RA	% Inhibition
Control	5	5.0 ± 0.8	0.3 ± 0.1[c]	94 ± 2[b]
1,25D	5	82.1 ± 17.4	33.8 ± 9.9[c]	60 ± 4[b]
PTH	3	50.6 ± 15.8	23.2 ± 14.5	62 ± 13[a]

Values are means ± SEM for individual experiments.
KEY: MNC, mononuclear cells; PTH, parathyroid hormone; TRAP, tartrate-resistant acid phosphatase; 1,25D, 1,25-vitamin D_3.
Significant inhibition by EP4RA at [a] $p < 0.05$, [b] $p < 0.01$.
Significant effect of EP4RA at [c] $p < 0.05$.

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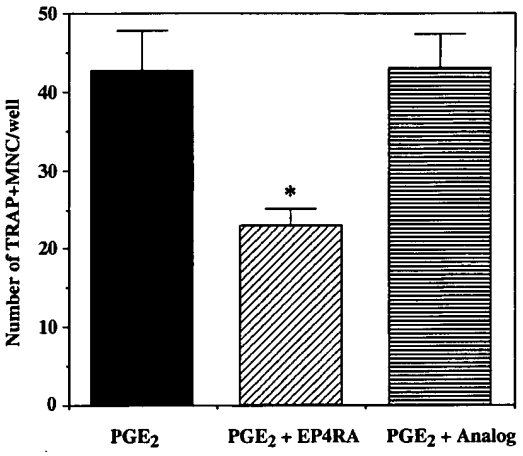

Figure 4. Effects of EP4RA and its inactive analog on $TRAP^+$ MNC formation in bone marrow culture. Bone marrow cells were treated with PGE_2 (1 μmol/L) plus EP4RA (1 μmol/L) or analog (1 μmol/L). Columns are means and vertical lines (SEM; n = 6). *Significant effect of EP4RA ($p < 0.05$).

6-week-old C57B1/6 and neonatal C57B1/6 × 129 Sv mice stimulated with a low dose of PGE_2 (0.1 μmol/L). EP4RA inhibited control cAMP production by 22 ± 1% and PGE_2 stimulated production by 43 ± 3% (**Table 2**).

We examined the effect of PGE_2 and EP4RA on mRNA levels for RANKL and OPG in POb by northern analysis. PGE_2 (0.01–1.0 μmol/L) increased RANKL mRNA expression by about 50% and EP4RA decreased RANKL mRNA expression in both control and PGE_2-treated culture at 24 h, although the effect was small at the highest concentration of PGE_2 (**Figure 6**). On the other hand, PGE_2 decreased OPG mRNA expression by 33%–50%, whereas EP4RA had no effect (Figure 6). The inactive analog did not alter RANKL or OPG mRNA expression (data not shown).

The effects of EP4RA on resorption were examined in cultured fetal rat long bones. EP4RA reduced ^{45}Ca release significantly by 12% at 2 days and by 25% at 5 days in control cultures.[45] Ca release stimulated by PGE_2 (1 μmol/L) was reduced significantly by 22% at 2 days and 37% at 5 days of culture (**Table 3**). The inactive analog had no significant effect.

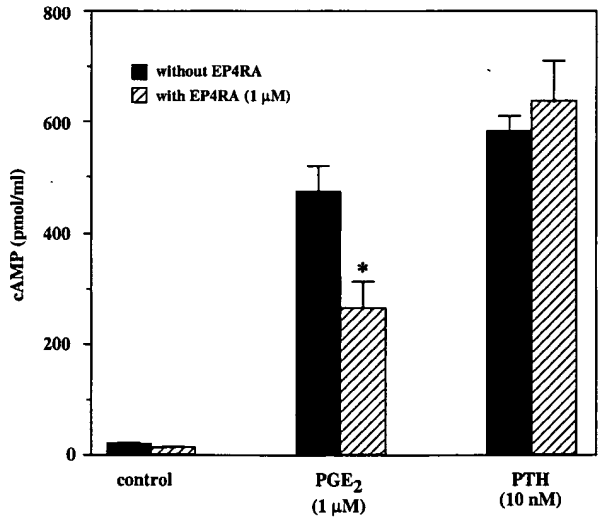

Figure 5. Effects of EP4RA on cAMP production in primary osteoblastic cells (POB). Primary osteoblasts were treated with IBMX with or without EP4RA for 20 min, followed by PGE_2 (1 μmol/L) or PTH (10 nmol/L). Columns are means and vertical lines (SEM; n = 3). *Significant effect of EP4RA ($p < 0.05$).

Table 2. Effects of EP4RA (1 μmol/L) on cyclicAMP (cAMP) production in primary osteoblastic cells stimulated by prostaglandin estradiol (PGE_2, 0.1 μmol/L)

Treatment	Number of experiments	cAMP (pmol/mL) without EP4RA	cAMP (pmol/mL) with EP4RA	% Inhibition
Control	2	21 ± 10	16 ± 8	22 ± 1[a]
PGE_2	3	126 ± 7	72 ± 4	43 ± 3[a]

Values are means ± SEM for individual cultures for cAMP levels and for individual experiments for inhibition.
[a]Significant inhibition by EP4RA at $p < 0.05$.

Discussion

The present study was undertaken to further define the role of the EP4 receptor in the stimulation of bone resorption by PGE_2. The results clearly demonstrate that a selective antagonist for this receptor, EP4RA, can inhibit osteoclastogenesis, decrease the stimulation of RANKL mRNA expression by PGE_2, and decrease osteoclastic bone resorption in fetal rat long bone organ cultures. The effect of EP4RA was greatest when low concentrations of PGE_2 were used. In addition, EP4RA could diminish the osteoclastogenic response to PTH and 1,25D. The number of osteoclasts produced in control, unstimulated cultures was also decreased in some experiments indicating that this osteoclast formation may have depended on endogenous prostaglandin production. The fact that EP4RA caused only partial inhibition of osteoclast formation and activity was most consistent with a dual receptor mechanism (see later), but could also be attributed to partial or incomplete block of the EP4 receptor. However, we found no significant difference in inhibitory effects with 0.1 or 1.0 μmol/L concentrations of EP4RA. Higher concentrations caused cellular toxicity (data not shown).

Recent studies using receptor knockouts and selective agonists have suggested roles in bone resorption for EP2 and EP4 receptors, both of which stimulate adenylylcyclase and increase cAMP production.[7,9,12,15,17] Tissues in which these receptors have been knocked out by homologous recombination have shown decreased

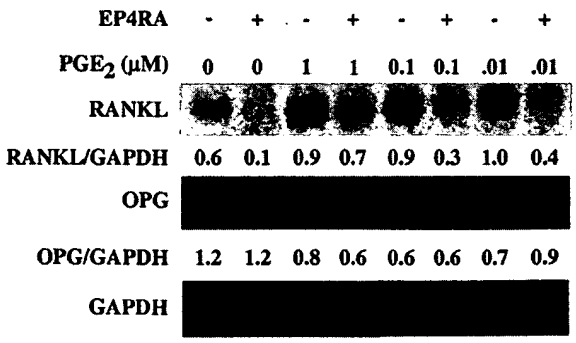

Figure 6. Effects of EP4RA on RANKL and OPG mRNA expression in POb by northern analysis. Cells were treated with PGE_2 (0.01–1 μmol/L) and with or without EP4RA (1 μmol/L) for 24 h. RANKL/GAPDH and OPG/GAPDH are the ratio of RANKL or OPG to GAPDH by densitometric analysis.

Table 3. Effects of EP4A (1 μmol/L) on release of previously incorporated ^{45}Ca from cultured fetal rat long bones stimulated by PGE_2 (1 μmol/L) with or without EP4A (1 μmol/L) or analog (1 μmol/L)

		2 days		5 days	
Treatment	n	^{45}Ca release	T/C ratio	^{45}Ca release	T/C ratio
Control	13	16.7 ± 0.6		30.8 ± 3.1	
Control + EP4RA	14	14.7 ± 0.4[b]	0.88	23.1 ± 1.1[b]	0.75
Control + analog	8	15.0 ± 0.7	0.90	27.5 ± 0.9	0.89
PGE_2	14	23.0 ± 0.8[a]	1.38	54.2 ± 5.7[a]	1.76
PGE_2 + EP4RA	14	17.9 ± 0.7[a,b]	1.07	33.8 ± 2.4[a,b]	1.10
PGE_2 + analog	8	23.6 ± 2.8[a]	1.41	50.4 ± 8.7[a]	1.64

Values are means ± SEM for ^{45}Ca release levels for the individual number of cultures (n). T/C ratio is the ratio of the treated cultures to the control.
[a]Significant effect of PGE_2 at $p < 0.05$.
[b]Significant effect of EP4RA at $p < 0.05$.

osteoclastogenic responses.[7,9,15] Selective agonists for EP2 and EP4 receptors can both increase cAMP production and stimulate bone resorption, and appear to have additive effects.[17] The precise mechanisms by which the two receptors enhance bone resorption have not been fully clarified. The fact that EP4RA is most effective against low concentrations of PGE_2 fits with studies using cells transfected with individual PGE_2 receptors, showing that EP4 has a greater affinity for PGE_2 than does the EP2 receptor.[1] Another apparent difference between the two receptors is that not only the osteoblasts, but also the hematopoietic cells from EP2 knockout animals, appeared to show diminished osteoclastogenic responses, whereas, in studies of EP4 knockout animals, only the osteoblasts showed a defect.[7,15] Our finding that the addition of EP4RA during the first 3 days of culture was almost as effective as continuous addition, while treatment from days 4–7 was less effective, has two possible explanations. The most likely is that EP4RA is critically involved in the initial steps of differentiation of the osteoblast to express RANKL and stimulate osteoclastogenesis. An alternative explanation is that, during the latter part of the culture, PGE_2 has an inhibitory effect on osteoclasts. This inhibitory effect has been attributed to an EP4 receptor, although EP2 may also be involved because the EP2-selective agonist, Butaprost, was shown to inhibit isolated osteoclasts.[8] Thus, when added during the last 4 days of culture, EP4RA might prevent this inhibition.

The effectiveness of EP4RA should prove useful in further elucidating the relative contributions of different receptor pathways in the responses of bone cells to PGE_2. Moreover, the fact that this agent can inhibit bone resorption may point to a new approach for the therapy of bone loss, not only in inflammatory disorders where prostaglandins are particularly implicated, but possibly also in osteoporosis.

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Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation

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Bone remodeling, comprising resorption of existing bone and *de novo* bone formation, is required for the maintenance of a constant bone mass. Prostaglandin (PG)E_2 promotes both bone resorption and bone formation. By infusing PGE_2 to mice lacking each of four PGE receptor (EP) subtypes, we have identified EP4 as the receptor that mediates bone formation in response to this agent. Consistently, bone formation was induced in wild-type mice by infusion of an EP4-selective agonist and not agonists specific for other EP subtypes. In culture of bone marrow cells from wild-type mice, PGE_2 induced expression of core-binding factor α1 (Runx2/Cbfa1) and enhanced formation of mineralized nodules, both of which were absent in the culture of cells from EP4-deficient mice. Furthermore, administration of the EP4 agonist restored bone mass and strength normally lost in rats subjected to ovariectomy or immobilization. Histomorphometric analysis revealed that the EP4 agonist induced significant increases in the volume of cancellous bone, osteoid formation, and the number of osteoblasts in the affected bone of immobilized rats, indicating that activation of EP4 induces *de novo* bone formation. In addition, osteoclasts were found on the increased bone surface at a density comparable to that found in the bone of control animals. These results suggest that activation of EP4 induces bone remodeling *in vivo* and that EP4-selective drugs may be beneficial in humans with osteoporosis.

Bones undergo continuous remodeling through repeated cycles of destruction and rebuilding (1). This remodeling is mediated by the well balanced actions of osteoclasts, which resorb old bones, and osteoblasts, which form new bones. However, in the elderly, especially in postmenopausal women, the extent of bone resorption far exceeds that of bone rebuilding, resulting in osteoporosis and the associated increases in bone fragility and susceptibility to fractures (2). About 100 million people are estimated to suffer from this debilitating disease worldwide. Several drugs have been developed to treat osteoporosis, with most inhibiting bone resorption and only a few promoting bone formation (3). Such modulation of only one of the two processes in bone remodeling renders these drugs of limited efficacy in restoring the normal balance and bone mass.

Recently, significant advances have been made in our understanding of molecular mechanisms of osteoclast and osteoblast differentiation (4), but such knowledge has not been exploited fully to develop a drug that corrects the imbalance and restores normal bone remodeling. Prostaglandins (PGs) are a group of lipid mediators that are produced from arachidonic acid in a variety of tissues under various physiological and pathophysiological conditions and serve to maintain local homeostasis (5). Among them, PGs of the E type work bimodally in bone metabolism (6). PGE_2 potently induces bone resorption in bone organ cultures, whereas repeated injection of this compound *in vivo* induces bone formation in a variety of animals including humans. However, the use of PGE_2 as a therapeutic agent in the treatment of bone loss has been hindered by unwanted actions that systemically applied PGE_2 exerts in the body. It is not understood, either, the mechanism by which it induces bone formation or how this *in vivo* effect is related to its bone resorbing activity *in vitro*.

PGE_2 exerts its effects through interaction with specific cell surface receptors (5). Four subtypes of PGE receptors—EP1, EP2, EP3, and EP4—have been identified. These receptors are encoded by distinct genes and are expressed differentially in the body. With the use of homologous recombination, we have generated mice that lack each of the four EP subtypes individually (7–9). We also have screened compounds on a panel of the cloned receptors and developed drugs that act specifically at each EP subtype (10). With these tools, we now have investigated which EP subtype mediates the bone-forming activity of PGE_2 and how activation of this receptor induces bone formation. We have also used ovariectomized or immobilized rats as models of diseases with bone loss and examined the efficacy of EP-subtype-specific drugs in the treatment of this condition.

Materials and Methods

Mice. Mice deficient in each EP subtype (7–9) were backcrossed for more than five generations into C57BL/6CrSlc (Japan SLC, Hamamatsu, Japan). Males of the F2 progenies of N10 $EP1^{-/-}$ mice, N5 $EP2^{-/-}$ mice, and N5 $EP3^{-/-}$ mice were used. Because $EP4^{-/-}$ mice do not survive in the C57BL/6 background because of patent ductus arteriosus (8), survivors of F2 progenies in the mixed genetic background of 129/Ola × C57BL/6 were intercrossed and the resulting male survivors were used. Mice were treated according to the guidelines for the protection of experimental animals of Kyoto University and Ono Pharmaceutical.

Chemicals. DI-004, AE1–259, AE-248, and AE1–329, agonists for EP1, EP2, EP3, and EP4, respectively, were described (10). A new EP4 agonist, ONO-4819, methyl 7-[(1*R*, 2*R*, 3*R*)-3-hydroxy-2-[(*E*)-(3*S*)-3-hydroxy-4-(*m*-methoxymethylphenyl)-1-butenyl]-5-oxocyclopentyl]-5-thiaheptanoate (Patent Cooperation Treaty publish no. WO 00/03980), shows inhibition constant values of

Abbreviations: PG, prostaglandin; EP, PGE receptor; Cbfa1, core-binding factor α1; OVX, ovariectomized.

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Table 1. Histomorphometric parameters in the tibia of sham-operated rats and immobilized rats with or without ONO-4819 treatment

Parameter*	Sham-operated rats (n = 4)	Control immobilized rats (n = 6)	ONO-4819-treated immobilized rats (n = 5)
Bone volume (BV/TV), %	7.82 ± 0.50	1.04 ± 0.38	13.4 ± 2.51†
Bone formation rate (BFR/TV), %/year	120.02 ± 7.77	17.01 ± 7.78	187.21 ± 42.97†
Bone surface (BS/TV), $\mu m/\mu m^2 \times 10^3$	3.49 ± 0.26	0.60 ± 0.19	6.37 ± 0.81†
Osteoid volume (OV/TV), %	0.39 ± 0.08	0.05 ± 0.02	1.17 ± 0.30†
Osteoid surface (OS/BS), %	30.39 ± 5.44	22.45 ± 5.18	44.51 ± 5.22†
Mineralizing surface (MS/OS), %	138.86 ± 19.68	78.41 ± 21.52	64.03 ± 11.01
Osteoblast surface (Ob.S/BS), %	11.90 ± 3.28	9.29 ± 3.01	22.38 ± 4.43†
Osteoclast surface (Oc.S/BS), %	13.81 ± 0.94	19.87 ± 4.40	15.09 ± 2.93
Osteoclast number (N.Oc/BS), no./mm	2.35 ± 0.33	3.22 ± 0.86	2.11 ± 0.48
Mineral apposition rate (MAR), μm/day	2.46 ± 0.11	2.20 ± 0.75	2.86 ± 0.12
Mineralization lag time (Mlt), day	1.14 ± 0.18	0.71 ± 0.23	2.48 ± 0.50†

*Nomenclature and abbreviations are from ref. 16.
†, $P < 0.05$ vs. immobilized control rats.

0.7, 56, and 620 nM for radioligand binding to EP4, EP3, and EP2, respectively, and values of more than 10 μM for EP1 and receptors for PGD_2, $PGF_2\alpha$, PGI_2, or thromboxane A_2. This compound was administered as an inclusion complex with α-cyclodextrin.

Local Infusion of PGE_2 and EP Agonists. Eight-week-old mice with body weights of 22–25 g were anesthetized. Their right femora were exposed, and the periosteum was removed around their shaft 8 mm in length. A polyvinyl catheter was fixed distally to the exposed bone surface and was connected proximally to an Alzet 1002 miniosmotic pump (Alza) implanted s.c. in the back and containing PGE_2 or EP agonists in ethanol/propylene glycol (40:60, vol/vol). The infusion was performed at a rate of 0.25 μl/hr for 6 weeks, with reservoir replacement every other week. The mice then were killed. Soft x-rays of the femur were taken as described (11). The anteroposterior and lateral diameters of the femur were measured in a callus-containing region of the treated bone and in the corresponding region of the contralateral femur. Each bone volume was calculated assuming the horizontal section of the femur to be elliptical, and the volume of the callus was obtained by subtraction. Histology of the femur was analyzed as described (12).

Bone Marrow Cell Culture. Bone marrow cells were obtained from femora of 8-week-old mice and cultured in the medium containing PGE_2 or vehicle as described (13). The medium was replaced every 3 days. After 21 days, the cells were rinsed with PBS, fixed in a 1:1:1.5 solution of 10% formalin/methanol/water for 2 h, and stained with the von Kossa method for mineralization. Nuclei were counterstained with neutral red. Photographs were taken with transmitted light, and the black-stained area of the mineralized nodules was measured with NIH IMAGE.

Immunoblot was performed as described with 100 μg protein of cell lysates and antibodies to core-binding factor α1 (Cbfa1) (14). Bound antibodies were detected with ECL Plus reagents (Amersham Pharmacia). Northern blot analysis was performed with 0.6 μg of poly(A) RNA. Hybridization was performed with random-primed ^{32}P-labeled probes prepared, with the 0.8-kb Eco*RI*-Hind*III* fragment of mouse Cbfa1 cDNA as a template (15).

Administration of ONO-4819 to Ovariectomized Rats. Fifteen-week-old female Crj:CD(SD)IGS (IGS) rats were anesthetized, and both ovaries were removed. ONO-4819 was dissolved in saline and administered either by i.v. infusion through a catheter into the right jugular vein or by s.c. injection in the back. Infusion was performed at a rate of 4 ml/kg per hr for 2 h twice per day. Seventy days after surgery, rats were killed and both femora and the fourth lumbar body were isolated. The right femur was fixed in 10% formalin and subjected to analysis with Micro Focus X-ray-Computed Tomography (MCT-CB100MF; Hitachi, Tokyo). The density of cancellous bone was measured in the left femur in a 0.77-mm-thick slice at a distance of 3 mm from the epiphysial growth plate by peripheral quantitative-computed tomography (Stratec Medizintechnik, Pforzeim, Germany) with a voxel size of 0.12 mm at a tube voltage of 50 kV. The lumbar body was used for compression testing, which was performed with a bone compression machine (MZ-500D; Maruto, Tokyo).

Immobilized Rat Model and Histomorphometric Analysis of Bone Tissues. Five- to six-week-old male IGS rats were anesthetized, and the left sciatic and femoral nerves each were resected at a length of 10 mm. Sham-operated rats received a similar operation without nerve resection. The rats then were systematically infused i.v. either with vehicle or ONO-4819 for 2 h twice a day. For bone density measurement, the rats were killed after 14 days, and the density of trabeculae of the left tibia was examined 4 mm from the proximal end as described above. For histomorphometric analysis, the infusion of ONO-4819 at 100 ng/kg per min or vehicle for 2 h twice a day continued for 28 days. On days 24 and 27, the rats received injection with tetracycline and calcein, respectively. After sacrifice on day 29, the left tibia was isolated. The sagittal block of the metaphysis was obtained, incubated with the Villanueva bone stain for 7 days, dehydrated, and embedded in methylmethacrylate. Sections of 4-μm thickness were prepared and subjected to the analysis by using a microscope coupled to the computerized bone morphometry analysis system (Luzex F Bone system, NIRECO, Tokyo). The primary parameters were measured as recommended (16) in an area between 1 and 1.2 mm from the growth plate and 200 μm apart from the cortical bone and were used to calculate the secondary parameters shown in Table 1.

Statistical Analysis. Data are presented as mean ± SE and were analyzed by using either Student's t test or, after variation analysis, Welch's t test or the Dunnett multiple comparison test. An associated probability (P value) of <0.05 was considered significant.

Results

Lack of PGE_2-Induced Bone Formation in $EP4^{-/-}$ Mice. By using a miniosmotic pump, we continuously infused PGE_2 into the periosteal region of the femur of wild-type C57BL/6 mice or

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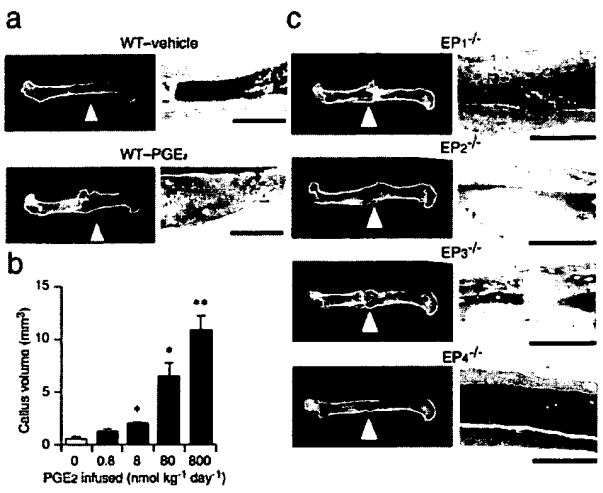


Fig. 1. Absence of PGE_2-induced bone formation in $EP4^{-/-}$ mice. (*a*) PGE_2-induced bone formation in wild-type mice. Typical radiographs (*Left*; arrowheads indicate the site of infusion) and histological preparations (*Right*; bars = 1 mm) of eight mice injected with vehicle or with PGE_2 at a dose of 800 nmol/kg per day are shown. (*b*) The dose dependence of the effect of PGE_2 on callus formation in wild-type mice. Data are values from six mice per dose. *, $P < 0.01$; **, $P < 0.001$ vs. vehicle-treated control mice. (*c*) The effect of PGE_2 on bone formation in EP-deficient mice. Typical radiographs (*Left*) and hematoxylin/eosin staining (*Right*) of the treated femur from each mouse strain infused with PGE_2 at a dose of 800 nmol/kg per day for 6 weeks are shown. Six mice per each strain were used in the analysis with reproducible results. (Bars = 1 mm.)

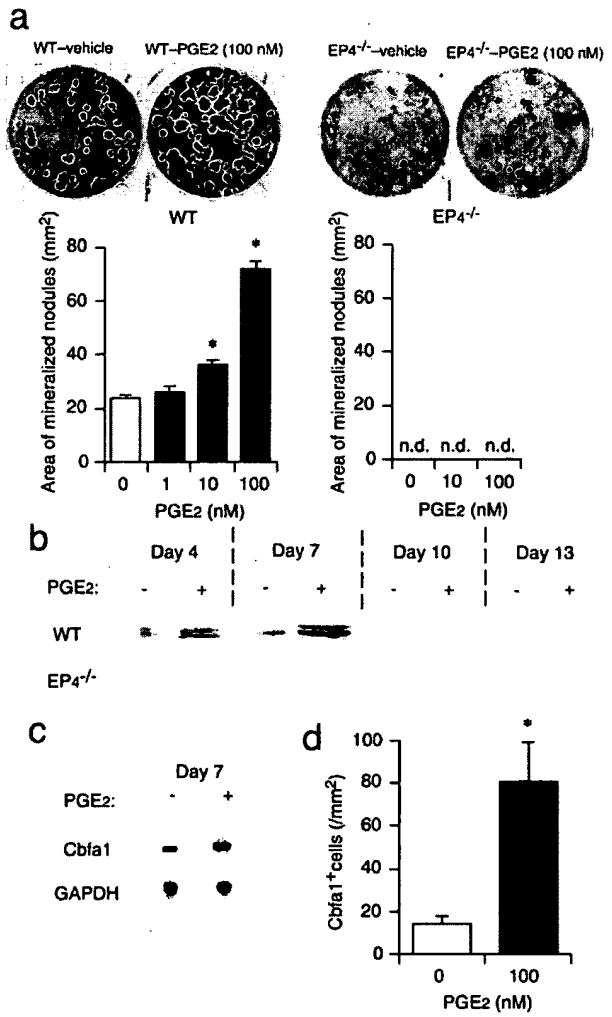


Fig. 2. EP4 mediates mineralized nodule formation and Cbfa1 expression in cultured bone marrow cells. (*a*) EP4-mediated enhancement of mineralized nodule formation by PGE_2. (*Upper*) von Kossa staining of mineralized nodules in bone marrow cell culture from wild-type C57BL/6 (WT) and $EP4^{-/-}$ mice in the presence of either vehicle or 100 nM PGE_2. Nuclei were counterstained with neutral red in the $EP4^{-/-}$ cell cultures. Typical results of six independent cultures are shown. (*Lower*) The concentration dependence of the effect of PGE_2 on mineralized nodule formation. Data are values from six experiments. *, $P < 0.001$ vs. vehicle-treated control. n.d., not detected. (*b*) Immunoblot analysis for Cbfa1 expression in bone marrow cells. Results of wild-type (WT) and $EP4^{-/-}$ cells cultured for indicated days in the absence or presence of 100 nM PGE_2 are shown. (*c*) Northern blot analysis for Cbfa1 mRNA abundance. Wild-type bone marrow cells cultured in the absence or presence of 100 nM PGE_2 for 7 days were subjected to Northern blot with probes specific to Cbfa1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. (*d*) Effect of PGE_2 on the number of Cbfa1-positive cells. Bone marrow cells from wild-type mice cultured for 4 days in the absence or presence of 100 nM PGE_2 were stained with anti-mouse Cbfa1 antibody and visualized by the ABC method. The number of positive cells per culture was determined ($n = 10$). *, $P < 0.001$ vs. vehicle-treated culture.

mice deficient in each EP subtype. After 6 weeks, the femur was isolated and bone formation was examined by both radiographic and histological analyses. Radiography revealed that PGE_2 induced extensive callus formation on the femur at the site of infusion in wild-type mice (Fig. 1*a*). The extent of callus formation depended on the dose of PGE_2 between 0.8 and 800 nmol/kg per day (Fig. 1*b*). Histological analysis showed marked thickening of the femoral cortex, with a large amount of woven bone as well as substantial accumulation of cells and bone matrix (Fig. 1*a*). Little bone formation was evident in the wild-type mice infused with vehicle alone. When PGE_2 was infused to mice deficient in each EP receptor subtype, the callus formation of as much extent as that seen in wild-type mice was observed in the cortex of the femora of $EP1^{-/-}$, $EP2^{-/-}$, and $EP3^{-/-}$ mice (Fig. 1*c*). This effect again depended on the doses of PGE_2 (data not shown). In contrast, no callus formation was detected in $EP4^{-/-}$ mice. On histology, massive formation of woven bone, similar to that apparent in wild-type animals infused with PGE_2, was seen in PGE_2-treated $EP1^{-/-}$, $EP2^{-/-}$, or $EP3^{-/-}$ mice but not in $EP4^{-/-}$ mice.

PGE_2 Induces Osteoblast Differentiation *in Vitro* Through EP4 Receptor Activation. The bone-forming activity of PGE_2 can be evaluated *in vitro* in a primary culture of rat bone marrow cells by measuring the formation of mineralized nodules (13). By application of this system to mice, we examined the mechanism of EP4-induced bone formation. Bone marrow cells were harvested from either wild-type C57BL/6 mice or $EP4^{-/-}$ mice and were cultured with PGE_2 for 3 weeks. Mineralized nodules were stained black by the von Kossa method, and their areas were summed. Culture of wild-type cells with vehicle alone resulted in the formation of mineralized nodules, and this was enhanced by the addition of PGE_2 in a concentration-dependent manner (Fig. 2*a*). In contrast, neither the basal level of mineralization nor its enhancement by PGE_2 was detected in cell culture derived from $EP4^{-/-}$ mice. These results suggested that PGE_2 might promote osteoblast differentiation from precursor bone marrow cells. To test this possibility, we examined the effect of PGE_2 on the expression of Cbfa1 in these cells. Cbfa1 is an osteoblast-specific transcription factor and an important determinant of osteoblast differentiation (4, 17). Immunoblot analysis with mouse mAb

αA8G5 to Cbfa1 (14) detected two bands of ≈65 kDa in lysates prepared from vehicle-treated, wild-type cells on day 4 or 7 (Fig. 2*b*). The amounts of these proteins were markedly increased, and the duration of their expression was prolonged in cells cultured with 100 nM PGE_2. In contrast, only a very little amount of Cbfa1 was detected in vehicle-treated $EP4^{-/-}$ cells, and the abundance was not enhanced by the addition of PGE_2. These results suggest that PGE_2 increases the amount of Cbfa1 via EP4. This effect of PGE_2 appears to be exerted at the mRNA level, given that Northern blot analysis revealed that culture of wild-type cells with PGE_2 increased the level of Cbfa1 mRNA (Fig. 2*c*). Immunostaining of wild-type cultures with anti-Cbfa1 antibody revealed that incubation with PGE_2 induced a significant increase in the number of cells containing Cbfa1 immunoreactivity (Fig. 2*d*), indicating that PGE_2 increased the number of Cbfa1-expressing cells and not simply increased the amount of Cbfa1 in a fixed number of cells. These results taken together identify EP4 as the EP receptor subtype that mediates the bone-forming activity of PGE_2, and suggest that EP4 achieves this effect through induction of osteoblast differentiation.

An EP4-Selective Agonist Potently Stimulates Bone Formation *in Vivo*. To corroborate these observations pharmacologically, we next applied agonists DI-004, AE1–259, AE-248, and AE1–329, which specifically target EP1, EP2, EP3, and EP4, respectively (10). Wild-type mice were infused continuously with one of these compounds for 6 weeks, and their *in vivo* bone-forming activities were assessed. Infusion of the selective EP4 agonist, AE1–329, markedly increased bone formation, as detected both radiographically and histologically, in a dose-dependent manner (Fig. 3 *a* and *b*). In contrast, the femora of mice infused with each of the agonists specific for the other three EP subtypes did not appear to differ from those of animals infused with vehicle (Fig. 3 *c–e*). We also tested the *in vitro* activities of these agonists on the formation of mineralized nodules in primary cultures of bone marrow cells. AE1–329 again was the only one of four compounds capable of inducing mineralized nodule formation (data not shown).

Administration of an EP4 Agonist Prevents Bone Loss and Restores Bone Mass and Strength in Rats Subjected to Ovariectomy and Immobilization. Given that the EP4 agonist is a potent inducer of bone formation in healthy animals, we next investigated whether this type of drugs might exert a similar action in animals with bone loss and, thereby, restore bone mass and strength. To this end, we modified AE1–329 to increase its chemical stability (Fig. 4*a*). The resulting compound, ONO-4819, was administered to ovariectomized (OVX) rats, a model for human postmenopausal osteoporosis. Both ovaries were removed from 15-week-old female rats, and the effect of daily administration of ONO-4819 was examined. Seventy days after ovariectomy, vehicle-treated control OVX rats exhibited marked osteoporosis, as was apparent from the almost complete loss of bone trabeculae (Fig. 4*b*). s.c. injection of ONO-4819 three times per day beginning on the day of ovariectomy inhibited bone loss in a dose-dependent manner (Fig. 4 *b* and *c*); at a dose of 10 μg/kg, the drug completely prevented the bone loss. Importantly, the full restoration of bone density also was obtained when the injection was initiated 20 days after ovariectomy. We next evaluated the effect of the EP4 agonist on bone strength by subjecting the fourth lumbar body from these animals to the compression test. Ovariectomy resulted in a marked reduction in bone strength. This fragility was prevented by treatment of animals with ONO-4819 (Fig. 4*d*). In animals injected with ONO-4819 at 30 μg/kg three times per day or infused at 100 ng/kg per min for 2 h twice per day, bone strength was significantly greater than that in vehicle-treated OVX rats. Histology of bones revealed that trabeculae similar in architecture and appearance to those found in the sham-operated rats and lined with osteoblasts were formed in the OVX rats treated with ONO-4819 (Fig. 4*e*).

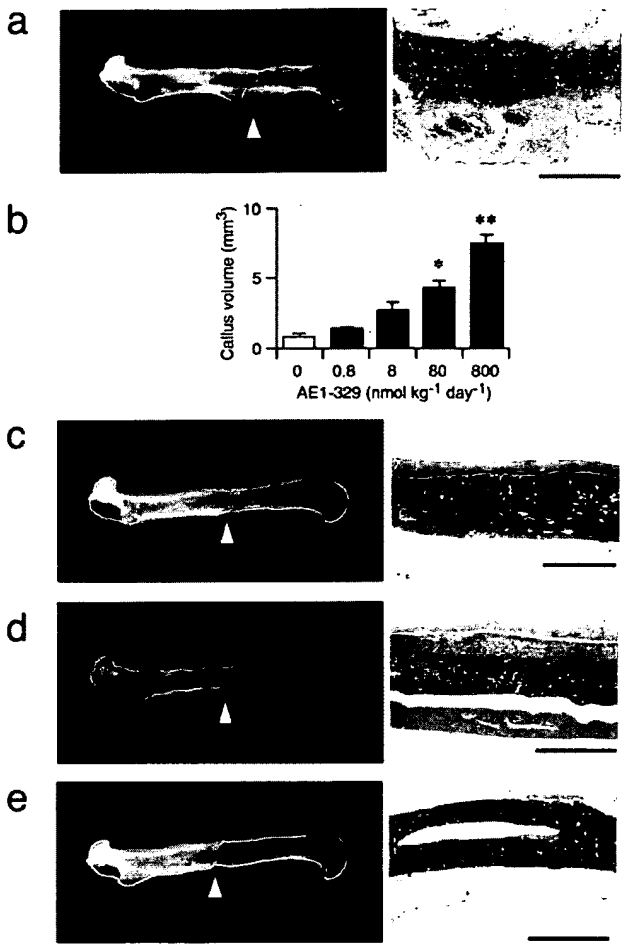
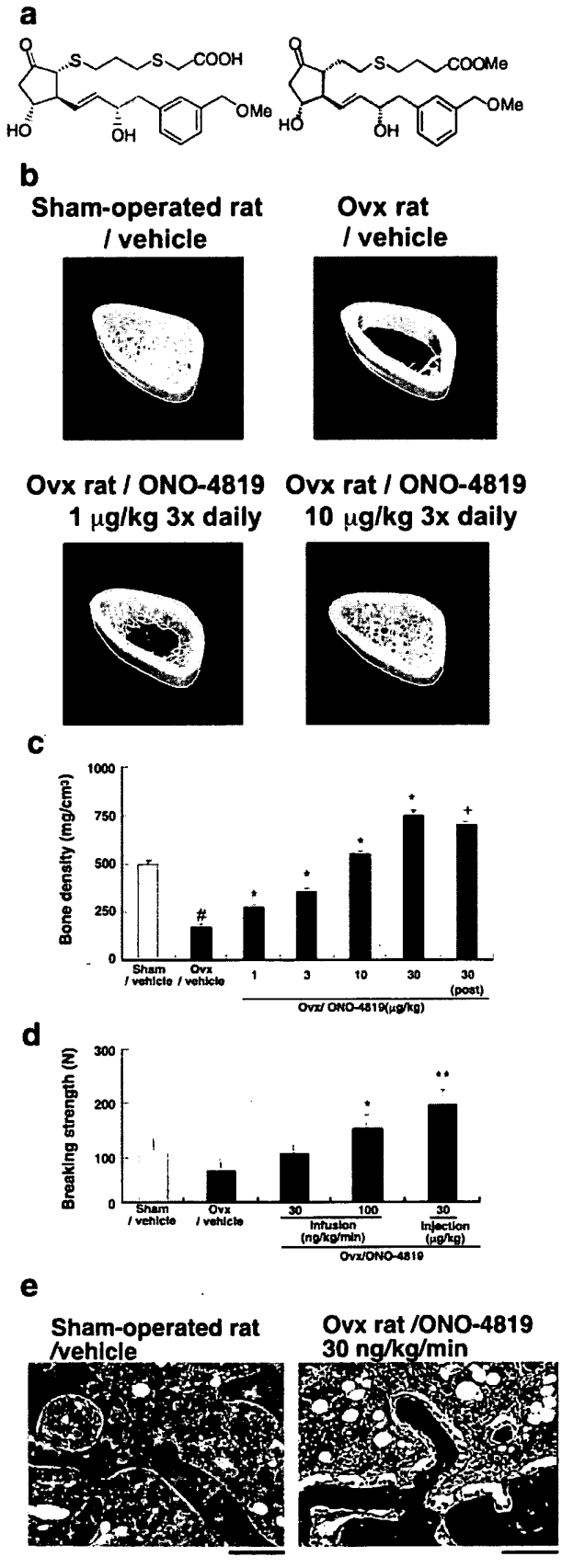


Fig. 3. Selective induction of bone formation by an EP4 agonist. Radiograph and histology of the femora treated with either the EP4 agonist AE1–329 (*a*), EP1 agonist DI-004 (*c*), EP2 agonist AE1–259 (*d*), or EP3 agonist AE-248 (*e*) are shown. Typical findings with a dose of 800 nmol/kg per day are shown. (Bars = 1 mm.) In *b*, the dose dependence of the effects of AE1–329 is shown. Data are from four animals per each dose. *, $P < 0.01$; **, $P < 0.001$ vs. vehicle-treated control.

In healthy animals, bone remodeling is stimulated by the mechanical tension applied to each bone as a result of its daily use, and prolonged immobilization results in a reduction in bone mass and deterioration of bone architecture. We therefore examined the effect of ONO-4819 on bone loss of immobilized rats. Immobilization of a hind limb induced a significant reduction in bone density of the tibia of the affected leg in 2 weeks. The infusion of ONO-4819 inhibited this reduction in a dose-dependent manner, preventing it completely at higher doses tested (Fig. 5). We next used rats subjected to this model and performed histomorphometric analysis on the effects of the ONO-4819 treatment (Table 1). Because the immobilization in our model affected significantly the cancellous bone and not the cortical bone, we limited our description to the changes in the cancellous bone of the metaphysis. This analysis revealed significant reduction in the volume of cancellous bone in immobilized rats as determined by bone volume (BV/TV). The ONO-4819 infusion significantly increased the bone volume including the osteoid volume and completely restored it in the immobilized



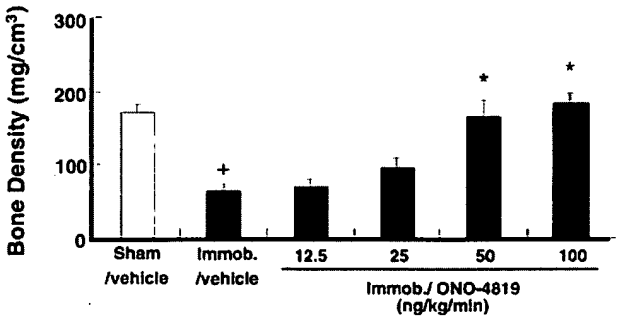


Fig. 5. Effects of ONO-4819 on immobilization-induced bone loss. Rats immobilized in the left hind limb (Immob) were infused systematically for 2 h twice per day with vehicle or the indicated doses of ONO-4819. After 14 days, the left tibia was isolated and subjected to analysis of bone density (n = 9–14 per group). +, $P < 0.01$ vs. the sham-operated group; *, $P < 0.01$ vs. control vehicle-treated immobilized group.

animals. This was reflected by the significant increase in bone formation rate in the ONO-4819-treated animals. The significant increases in bone surface (BS/TV) and in the bone surface lined with osteoblasts (Ob.S/BS) were also noted. These findings together with a similar density of osteoclasts on bone surface (N.Oc/BS) in ONO-4819-treated rats indicate that the total number of osteoclasts in the tissue markedly increased with the increase in the bone surface. On the other hand, the calcification rate as determined by double fluorescence labeling (mineral apposition rate) was not affected by this treatment.

In the above experiments, ONO-4819 administration caused no significant change in the serum concentrations of alkaline phosphatase, calcium, and inorganic phosphate in the animals (data not shown). Ectopic calcification in tissues such as cardiac valves or aorta was not found. No significant change in the blood pressure was detected in conscious rats by either infusion or injection of the drug at the doses described, although the infusion of the highest dose (100 ng/kg per min i.v.) induced less than 20% elevation of the heart rate.

Discussion

In this study, with both genetic and pharmacological approaches, we have unambiguously identified EP4 as the receptor that

Fig. 4. Prevention of bone loss and restoration of bone mass and strength by the EP4 agonist ONO-4819 in OVX rats. (*a*) Structure of AE1-329 (*Left*) and ONO-4819 (*Right*). (*b*) Bone architecture of OVX rats treated with ONO-4819. Seventy days after surgery, the right femur was isolated from a sham-operated rat as well as from OVX rats injected either with vehicle or two different doses of ONO-4819 and was subjected to analysis by x-ray-computed tomography. The three-dimensional structure was constructed by piling up images, and typical architecture of the metaphysis of each bone is presented. (*c*) Effects of ONO-4819 on bone density in OVX rats. OVX rats were injected s.c. with either vehicle or the indicated doses of ONO-4819 three times per day, either beginning on the day of surgery or day 20 after ovariectomy (post). Seventy days after the surgery, the left femur was isolated and subjected to analysis of bone density (n = 8 per group). #, $P < 0.05$ vs. the sham-operated group; *, $P < 0.05$ vs. control vehicle-treated OVX group (Dunnett test); +, $P < 0.05$ vs. control vehicle-treated OVX group (Student's t test). (*d*) Effects of ONO-4819 on bone strength in OVX rats. OVX rats were treated either by i.v. infusion of ONO-4819 at a rate of 30 or 100 ng/kg per min (≈15 and 50 nmol/kg per day) or by s.c. injection of the drug at a dose of 30 μg/kg three times per day (≈200 nmol/kg per day). Seventy days after surgery, rats were killed, and the fourth lumbar body was isolated and subjected to a compression test. No significant difference in the size of the bone was detected among the groups. Data are from eight rats per group. *, $P < 0.05$ vs. control vehicle-treated OVX group; *, $P < 0.01$ vs. control vehicle-treated OVX group. (*e*) Histology of the bone. Hematoxylin/eosin staining of the decalcified transverse sections of the epiphysis of the femur of sham-operated rats or OVX rats with ONO-4819 infusion is shown. (Bars = 100 μm.)

mediates the bone-forming activity of PGE_2. The role of this receptor in bone formation was suggested previously only indirectly by the use of a limited repertory of EP-acting compounds (18). We have found that activation of EP4 induced callus formation on the femur of mice and restored the volume of cancellous bone in OVX or immobilized rats. The histomorphometric analysis in the latter model revealed increases in both the total volume of the bone and the volume of osteoid, suggesting that these effects of the EP4 activation are exerted by *de novo* bone formation. *In vitro* in the bone marrow cell culture, EP4 activation increased the number of Cbfa-1-positive cells, suggesting that EP4 exerts such an effect by inducing osteoblast differentiation. Consistently, the density of osteoblasts lining the bone surface (Ob.S/BS) increased with ONO-4819 treatment in the *in vivo* models. It is noteworthy that the callus induced by PGE2 in mice contained many fibrous tissues and that the bone of ONO-4819-treated rats showed a mineralizing surface (MS/OS) of about half of that seen in sham-operated animals. These results indicate that the osteoblasts induced by EP4 activation produce bone matrix at a rate exceeding that of calcification. This was reflected by the prolonged mineralization lag time (Mlt) with an unchanged mineral apposition rate. However, the trabeculae formed were well connected, and the majority was sufficiently calcified as shown in Fig. 4*e* and Table 1, yielding substantial strength to the bone of OVX animals (Fig. 4*d*).

Our current study thus suggests that EP4 activation induces osteoblasts and thereby stimulates *de novo* bone formation. Previously, we also noted in bone organ culture that EP4 in mature osteoblasts mediates PGE_2-induced osteoclast differentiation (19, 20). We wondered how these two EP4 actions are coordinated *in vivo* in the bone of animals treated with the EP4 agonist. The bone morphometric analysis has shown that the EP4 agonist did not decrease the density of osteoclasts despite the increase in the bone surface, suggesting that it increased the number of osteoclasts in parallel with the *de novo* increase in bone. It is tempting to speculate that the PGE_2-EP4 signaling first works in osteoblast precursors to induce osteoblasts for bone formation and then works in mature osteoblasts for induction of osteoclasts on newly formed bones. Both EP4 and EP2 respond to PGE_2 and are coupled to activation of adenylate cyclase. Both also are implicated in PGE_2-induced osteoclastogenesis (19–21). It is interesting in this respect that we have not observed any involvement of EP2 in PGE_2-induced bone formation in mice (Figs. 1 and 3). We neither have observed any bone-forming effects of the EP2-selective agonist in OVX rats (data not shown). Although we cannot exclude a redundant role of EP2 in other species, these results indicate that EP4 is the only system mediating PGE_2-induced bone formation at least in rodents. It is curious, therefore, that the skeleton of $EP4^{-/-}$ mice either alive to adulthood or dead in the neonatal period is apparently normal (19, 20), suggesting that some pathway(s) other than the PGE_2 system works for physiological maintenance of bone. It remains to be clarified in what physiological or pathological context the PGE_2-EP4 signaling is mobilized for bone formation.

Several types of drugs currently are used for the treatment of bone loss (3). These drugs either inhibit differentiation and functions of osteoclasts or activate osteoblasts. However, none of these drugs are able to restore the balance between bone formation by osteoblasts and bone resorption by osteoclasts. PGE_2-EP4 appears to induce both osteoblastogenesis and osteoclastogenesis and to integrate the two actions temporarily and spatially *in situ* in bone remodeling. In this respect, the action of PGE_2 may be similar to that of PTH, which also promotes both bone formation and resorption (1, 3, 22). Recently, clinical efficacy of PTH for postmenopausal osteoporosis has been reported (23). On the other hand, the numerous, unwanted effects of PGE_2 injected systematically have precluded its use in therapeutics for bone loss. Because EP4 agonists are selective to only one subtype of EP receptors, they are expected to avoid several adverse actions caused by systemic administration of PGE_2. Indeed, ONO-4819 lacks the uterine-contracting activity of PGE_2. Furthermore, administration of ONO-4819 in rodents does induce diarrhea, hypotension, and thickening of intestinal epithelium but at higher doses than that required for bone formation. However, given various differences between the rodent models and human patients, it may be too early to conclude that EP4 agonists exert beneficial effects in humans. Their therapeutic potential will be tested rigorously in future studies.

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